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Acetylene inhibition of Nitrogen Fixation by Klebsiella pneumoniae M5al

Robert L. Todd

ACETYLENE INHIBITION OF NITROGEN FIXATION BY *KLEBSIELLA PNEUMONIAE* M5a1

BY

ROBERT L. TODD

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RLT

ACETYLENE INHIBITION OF NITROGEN FIXATION

BY KLEBSIELIA PNEUMONIAE M5a1

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Thesis Adviser Date

Head, Bacteriology Department Date

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INTRODUCTION

The four most abundant elements needed to support plant growth are carbon, hydrogen, oxygen, and nitrogen. Of these carbon, hydrogen, and oxygen are available to the plant in ample supply from the atmosphere and soil. Nitrogen, however, is available only in limited quantities in a form useable to the plant. The nitrogen is usually supplied in the form of nitrates or ammonium-nitrogen. The largest supply of nitrogen is in the atmosphere but it is in the elemental, uncombined form. Few plants, if any, are able to convert, directly or indirectly, this atmospheric nitrogen to organic matter. Plants which assimilate elemental nitrogen are said to "fix" nitrogen. The term nitrogen fixation will be used in this context.

One group of "plants" which are capable of fixing nitrogen are certain species of bacteria. Most of the information concerning the mechanism of bacterial nitrogen fixation has been obtained in the last few decades. Workers in this area have turned to various areas in their study of the mechanism of nitrogen fixation. Some of these include: physiology, metabolic pathways, cell-free fixation, enzyme induction and repression, inhibitors, and the study and isolation of co-factors or combination of these studies.

This study was, therefore, undertaken to extend the knowledge of the mechanism of nitrogen fixation by a nonsymbiotic nitrogen fixing bacterium, Klebsiella pneumoniae. The effect of acetylene gas (which has a structure closely analogous to nitrogen gas) on the nitrogen fixation pathway of K. pneumoniae was the subject of this study.

LITERATURE REVIEW

Microorganisms that can fix atmospheric nitrogen nonsymbiotically may be divided into two classes: heterotrophic and photoautotrophic. Nitrogen fixation by heterotrophic bacteria was first reported in the anaerobic bacterium, Clostridium, and in the aerobic, Azotobacter. Winogradsky (1893) was first to cultivate Clostridium in a nitrogen free medium. Azotobacter was described by Beijerinck and van Delden (1902) and was shown to fix nitrogen only in a mixed culture. Later workers (Fischer, 1949) have shown that pure cultures of Azotobacter can fix nitrogen.

Photoautotrophic organisms of the class Myxophyceae were noted to grow in a medium with limited nitrogen by Beijerinck and van Delden (1902). Nitrogen fixation by these blue-green algae was confirmed by Drewes in 1928 and by Fogg in 1942 (Thimann, 1963). It was reported by Gest et al. (1950) that nitrogen gas, as well as ammonium, inhibited the photosynthetic reactions of Rhodospirillum, a nonsulfur purple bacterium. It was this observation that led them to postulate Rhodospirillum might be able to fix nitrogen. Gest et al. (1956) using labeled nitrogen gas, proved the nitrogen fixing ability of this organism.

In addition to the bacteria, Duggar (1916) reported nitrogen fixation in the fungi. Metcalfe and Chayen (1954) have reported that two yeasts, isolated from soil were capable of fixing measurable amounts of nitrogen.

To extend the knowledge of the mechanism of nitrogen fixation, workers in this area have turned to other bacteria capable of fixing nitrogen. This change was to serve as a comparison of similar traits with those of the well known nitrogen fixing bacteria. In the course of these investigations many new metabolic relationships between overall metabolism and nitrogen fixation were noted. An example of this can be found in Klebsiella pneumoniae (Aerobacter aerogenes). This organism is a facultative anaerobe that grows most abundantly in the presence of oxygen when it is supplied with a fixed form of nitrogen. It fixes only small amounts of nitrogen gas when it is grown aerobically. However, under anaerobic conditions it fixes nitrogen gas as well as those previously recognized nitrogen fixing bacteria (Hamilton and Wilson, 1955). For this reason this organism was used in studies of the mechanism of nonsymbiotic nitrogen fixation.

Review of Work Performed on Klebsiella pneumoniae (Aerobacter aerogenes)

Skinner (1928) was first to observe nitrogen fixation in the coliform group of bacteria. He referred to an organism isolated from sewage, Bacterium aerogenes, which was capable of fixing atmospheric nitrogen. Newton (1952) confirmed Skinner's earlier work on nitrogen fixation by the use of isotopic nitrogen. Bhat and Palacios (1949) noted that when soil was inoculated with Aerobacter aerogenes the total level of nitrogen increased. Nitrogen fixation by several strains of A. aerogenes was reported by Hamilton and Wilson (1955).

By culturing the organisms anaerobically in a well buffered medium sufficient nitrogen was fixed to be measured by the semi-micro Kjeldahl method of Wilson and Knight (1952). Hamilton and Wilson postulated the adaptive nature of the nitrogenase system in A. aerogenes by observing that minute quantities of fixed nitrogen must be added to initiate nitrogen fixation.

Jensen (1956) isolated several strains of A. aerogenes from water which he reported fixed nitrogen. He concluded that yeast extract must be added to the medium for fixation to occur. This suggested that some growth factor was supplied by yeast extract that was essential for nitrogen fixation.

Pengra and Wilson (1958) made a study of the physiology of nitrogen fixation by Aerobacter aerogenes using a strain M5a1 which Hamilton and Wilson (1955) had reported fixed molecular nitrogen. Pengra and Wilson (1958) used a growth medium which was a modification of one devised by Monod and Wollman (1947). They were able to demonstrate that fixation was inhibited by oxygen and by hydrogen and that hydrogen was a competitive inhibitor of nitrogen fixation. In addition, nitrogen fixation was shown to be inducible, with an induction time of about 15 hours. The mineral requirement for nitrogen fixation by A. aerogenes M5a1 was also studied by Pengra and Wilson (1959). They were able to show that iron and molybdenum were required for fixation, but that there was no calcium requirement. Yoch and Pengra (1964) concluded that magnesium is not required for

nitrogen fixation by A. aerogenes. However, they noted there is a definite requirement for magnesium in the metabolism of the organism.

Casein hydrolysate (Patil, 1963; Patil, Pengra and Yoch, 1967) exerts a stimulatory action on the induced formation of the enzyme(s) of the nitrogen fixing system of A. aerogenes. Thus, casein hydrolysate appears to supply stimulatory amino acids for the formation of nitrogenase (Yoch and Pengra, 1966).

Earlier workers, as noted, referred to the facultative anaerobic, Gram negative, nitrogen fixing rods as Aerobacter aerogenes (Pengra, 1964). Strains of the species of the tribe Klebsielleae were compared by serotypes and by the ability of the organism to fix molecular nitrogen (Mahl et al. 1965). It was concluded from this study that Aerobacter aerogenes strain M5al is more correctly classified as Klebsiella pneumoniae strain M5al. In this study the organism used will be referred to as K. pneumoniae strain M5al. This organism is the same as A. aerogenes strain M5al as referred to by Hamilton and Wilson (1955); Pengra and Wilson (1958, 1959); Patil (1963); Yoch and Pengra (1964); Yoch (1965); Patil, Pengra and Yoch (1967).

Cell-Free Nitrogen Fixation by Nonsymbiotic Bacteria

By far the greatest advancement in the study of the mechanism of nitrogen fixation was the advent of an active, cell-free nitrogen fixing preparation. The first cell-free system which consistently fixed nitrogen was prepared from Clostridium pasteurianum

(Carnahan et al., 1960). The system was prepared by autolyzing dried cells with phosphate buffer. Fixation was shown to be associated with pyruvate oxidation.

Schneider et al. (1960) obtained fixation from cell-free extracts of Rhodospirillum rubrum and from some blue-green algae. Their extracts were prepared by sonic disruption of the cells.

Nicholas and Fisher (1960) reported the first reproducible system of nitrogen fixation by extracts of Azotobacter vinelandii. Ultrasonic and lysozyme treatments were used as a method of cell breakage. Nimeck, Wilson and Nicholas (1963) used a bacteriophage to obtain cell-free extracts of Azotobacter vinelandii which were shown to fix nitrogen. They used an isotope of nitrogen (N^{15}) to validate their results.

Grau and Wilson (1963) working with Bacillus polymyxa, obtained a cell-free nitrogen fixing preparation using lysozyme to disrupt the cells. Lindsey (1963) reported that nitrogen fixing extracts of Klebsiella pneumoniae could not be made by autolysis of dried cells, by sonic disruption or by the use of lysozyme.

A cell-free nitrogen fixing extract of Klebsiella pneumoniae was reported in 1964 by Centifanto (Mahl, 1966). The organism of her study was originally classified as a Psychotria bacteriophila, a leaf nodule endophyte. She prepared the cell-free extracts with the French pressure cell. Activity was low compared to the other nitrogen fixing cells but she was able to show significant results by the use of isotopic nitrogen.

Bulen et al. (1964) obtained a reproducible cell-free nitrogen fixing system from extracts of Azotobacter vinelandii. Their extracts were prepared in a French pressure cell. An ATP generating system and a reductant were needed. Hydrogenase preparations of C. pasteurianum were first added as a reductant. This was later replaced with dithionite as the reductant.

Mahl and Wilson (1966) using the procedures of Bulen et al. (1964, 1965) obtained a cell-free nitrogen fixing system with extracts of Klebsiella pneumoniae M5al prepared in a French pressure cell.

Molecular Gases as Inhibitors of Nitrogen Fixation

I. Hydrogen

Wilson (1940), while studying symbiotic nitrogen fixation with Rhizobium trifolii, established that molecular hydrogen acts as a competitive inhibitor of nitrogen fixation. This was the first report of a molecular gas acting as an inhibitor of nitrogen fixation. Wyss and Wilson (1941) reported hydrogen inhibited nitrogen fixation by Azotobacter, a nonsymbiotic, aerobic, nitrogen fixing bacterium. Burris and Wilson (1946) obtained evidence that inhibition of nitrogen fixation by hydrogen could occur in the blue-green alga Nostoc muscorum. It soon became apparent that hydrogen would inhibit nitrogen fixation by all the known aerobic nitrogen fixing organisms.

Rosenblum and Wilson (1950) unsuccessfully attempted to prove that hydrogen acts as an inhibitor of nitrogen fixation for the anaerobic nitrogen fixing Clostridium pasteurianum. Shug et al. (1956) reported hydrogen to be an inhibitor of nitrogen fixation by C. pasteurianum. Westlake and Wilson (1959) presented evidence to further support the view that hydrogen is a specific, competitive inhibitor of nitrogen fixation by C. pasteurianum. Gest et al. (1950) could detect no hydrogen inhibition of nitrogen fixation by the photosynthetic nitrogen fixing bacteria, Rhodospirillum rubrum. However, hydrogen inhibition of nitrogen fixation by R. rubrum has been shown by Pratt and Frenkel (1959).

Hino and Wilson (1958) concluded that in the nitrogen fixation pathway of Bacillus polymyxa hydrogen acts as a competitive inhibitor. Pengra and Wilson (1958) extended this observation to include another facultative anaerobe, Aerobacter aerogenes. Using a species of Achromobacter which does not anaerobically evolve hydrogen gas, Goerz and Pengra (1961) reported definite inhibition of nitrogen fixation by hydrogen.

II. Carbon Monoxide

Lind and Wilson (1941) observed inhibition of nitrogen fixation in Azotobacter vinelandii with 0.2 per cent carbon monoxide in the atmosphere over their cultures. Burris and Wilson (1946) reported similar results for Nostoc muscorum. Virtanen et al. (1953) reported that the anaerobic nitrogen fixing system of C. pasteurianum was

80 per cent destroyed by a 0.3 per cent carbon monoxide atmosphere. Amounts of carbon monoxide needed for inhibition of nitrogen fixation by Aerobacter aerogenes have been reported (Pengra, 1958). He has shown that a 1.0 per cent atmosphere of carbon monoxide is needed to cause a 70 per cent inhibition of nitrogen fixation. Dilworth (1966) reported 0.01 atmosphere (one per cent) of carbon monoxide produced a 90 per cent inhibition of nitrogen fixation by C. pasteurianum.

III. Oxygen

Burk, in 1930, (Bradbeer and Wilson, 1963) made a quantitative investigation on the effect of oxygen on the growth of Azotobacter under nitrogen fixing conditions. He found growth was maximal at 2-4 per cent oxygen and decreased with both higher and lower partial pressures. Pratt and Frankel (1959) found that four per cent oxygen completely inhibits nitrogen fixation by R. rubrum. Nitrogen fixation by B. polymyxa is reported by Hino and Wilson (1958) to be significantly reduced by one per cent oxygen. Pengra and Wilson (1958) reported that nitrogen fixation by Aerobacter aerogenes is completely inhibited by a five per cent atmosphere of oxygen.

IV. Nitrous Oxide

Molnar et al. (1948) reported nitrous oxide to be a specific inhibitor for nitrogen fixation by A. vinelandii. Repaske and Wilson (1952) demonstrated that this inhibition was competitive. Virtanen and Lundbow (1953) extended these observations to C. pasteurianum and Hino (1955) to B. polymyxa.

V. Acetylene

The most recent molecular gas to be reported as an inhibitor of nitrogen fixation is acetylene. Schollhorn and Burris (1966) have reported that in extracts of Clostridium pasteurianum acetylene inhibits nitrogen fixation. They further report that the partial pressure required for inhibition by acetylene is comparable to that required by carbon monoxide. This inhibition was found to be competitive when a low partial pressure of acetylene was used. Similar results using cell-free extracts of C. pasteurianum have been demonstrated by Dilworth (1966). In addition, he has shown acetylene to be reduced to ethylene by nitrogen fixing extracts of C. pasteurianum. Koch and Evans (1966) have shown that soybean root nodules can reduce acetylene to ethylene. This reduction by the nitrogen fixing enzyme(s) would indicate from the data of these workers, that the system is nonspecific for the electron donor and the first step in the reduction of molecular nitrogen is a two electron reduction.

The purpose of this investigation is to study the relationship between molecular nitrogen and acetylene in the nitrogen fixation pathway of Klebsiella pneumoniae strain M5al with the hope that the mechanism of nonsymbiotic nitrogen fixation may be further clarified.

MATERIALS AND METHODS

Organism

The organism used throughout this study was Klebsiella pneumoniae strain M5al. This organism was originally isolated by Dr. E. McCoy, of the University of Wisconsin, from a 2-3 butanediol fermentation. This and other strains of K. pneumoniae (formerly known as Aerobacter aerogenes) were characterized by Hamilton and Wilson (1955). Pengra and Wilson (1958) studied the physiology of this organism. Other workers have used K. pneumoniae (A. aerogenes) in a variety of studies on the mechanics of nitrogen fixation by this facultative anaerobe. (Pengra and Wilson, 1959) (Patil, 1963) (Yoch and Pengra, 1964) (Yoch, 1965) (Yoch and Pengra, 1965) (Yoch and Pengra, 1966) (Patil, Pengra and Yoch, 1967).

The name of this organism has recently been the subject of controversy. On the basis of serotypes Mahl et al. (1965) reclassified A. aerogenes strain M5al as K. pneumoniae strain M5al. On the basis of their reported work the organism used in this study shall be referred to as K. pneumoniae strain M5al.

The culture was maintained at 5 C on nutrient agar slants to which 0.2 per cent yeast extract had been added. Stock cultures were transferred every two months. To check for purity the culture was periodically streaked on nutrient agar plates and Gram stains of the organism were observed microscopically.

Medium

The nitrogen-free salts medium which was used in this study to grow nitrogen-fixing cultures was originally developed by Pengra and Wilson (1958). Pengra and Wilson (1959) eliminated calcium from their medium. Patil (1963) found it necessary to incorporate a physiological concentration of sodium chloride to prevent clumping of the cells. This modified medium was used by Yoch and Pengra (1964), Yoch (1965), Yoch and Pengra (1965), Yoch and Pengra (1966), and Patil, Pengra and Yoch (1967).

Mahl (1966) further modified the medium to exclude magnesium. I believe this omission was an error in manuscript preparation because Mg is required for growth but not for nitrogen fixation (Yoch and Pengra, 1964).

The final composition of the medium used in this study was:

Solution I	
Na_2HPO_4	12.5 g.
KH_2PO_4	2.0 g.
Distilled Water	500 ml.
Solution II	
$MgSO_4.7H_2O$	0.2 g.
Fe-Mo solution (Wilson and Knight, 1952)	1.0 ml.
Sucrose	20.0 g.
Distilled Water	500 ml.

The two solutions were autoclaved at 121 C for 15 minutes, cooled and mixed just before use. All inorganic chemicals used in this study were obtained as an analytical or reagent grade.

Reagents

Phosphate buffer

Three parts of 0.25 M KH_2PO_4 were mixed with 7 parts 0.25 M Na_2HPO_4 (vol./vol.). The final pH was 7.1. The buffer was stored at 4 C until used.

Cacodylate buffer (Mahl, 1966)

The cacodylate buffer was prepared by dissolving either 1.01 g or 4.04 g sodium cacodylate-$3H_2O$ (K&K Laboratories, Plainview, N. Y.) with 100 ml distilled water which resulted in a 0.05 M or 0.2 M solution respectively.

Biuret Reagent (Clark, 1964)

One and one-half gram of copper sulfate ($5H_2O$) and 60 g sodium potassium tartrate were dissolved in 500 ml distilled water. To this was added 300 ml of 10 per cent sodium hydroxide. The total volume was brought to one liter with distilled water. The solution was stored at 4 C in a polyethylene bottle.

Ammonium chloride (stock solution)

The ammonium chloride solution (0.189 g per 100 ml distilled water) containing 500 g N/ml was sterilized by autoclaving at 121 C for 15 min and stored under refrigeration at 4 C.

Scintillation fluid

The detection fluid of Chase and Rabinowitz (1962) was prepared by dissolving 3 g of 2,5 diphenyloxazole (PPO) and 100 mg of 2,2[1] paraphenylene bis-5-phenyloxazole (POPOP) in 300 ml of toluene. The final volume was then adjusted to 1000 ml with toluene.

Gases

Elemental

All the gases (nitrogen, hydrogen, helium, and acetylene) used in this study were supplied by Midwest Welding Company, Watertown, South Dakota. All were supplied at the highest purity available. Pengra and Wilson (1958) reported that nitrogen fixation by Klebsiella pneumoniae M5al is completely inhibited by a 5 per cent atmosphere of O_2. Therefore the amount of O_2 contained in the acetylene used in this study was determined.

Samples of tank acetylene were assayed in an Aerograph Model A-350B Gas Chromotograph. The following ratio of gases was observed:

	Gas ratio	Per cent
Oxygen	0.495	0.1
Nitrogen	3.5625	0.8
Acetylene	454.8	99.1

The O_2 level in tank acetylene is far less than that needed for inhibition of nitrogen fixation.

Acetylene - 1,2 C^{14}

The acetylene - 1,2 - C^{14} was obtained from Nuclear Research Chemicals, Incorporated, Orlando, Florida. The gas was supplied in 50 μc vials at a specific activity of 50μc/mM. It was stored at -25 C until used to prevent the polymerization of the acetylene. The acetylene - 1,2 - C^{14} was used over a two-week period. The manufacturer recommends storage for up to four months at -25 C.

Protein Determinations

The Biuret assay method (Clark, 1964) was used as a method of determining the amount of protein in a sample. Two ml of sample was mixed with eight ml of Biuret reagent. A reagent blank was prepared which contained 2 ml water and 8 ml Biuret reagent. The mixtures were held for 30 minutes to allow for color development. The samples were then read in a Bausch and Lomb Spectronic 20 spectrophotometer at 550 mμ using 18 mm cuvettes. The protein values were read from a standard curve prepared from bovine albumin (Figure 1).

Procedure for Growth Studies

Inoculum

The procedures used in the growth studies are essentially those of Pengra and Wilson (1958). The organism Klebsiella pneumoniae M5al was carried on nutrient agar slants from which transfers were made into 250 ml flasks containing 50 ml of a nitrogen-free medium. An atmosphere of nitrogen gas was placed over each culture. When the

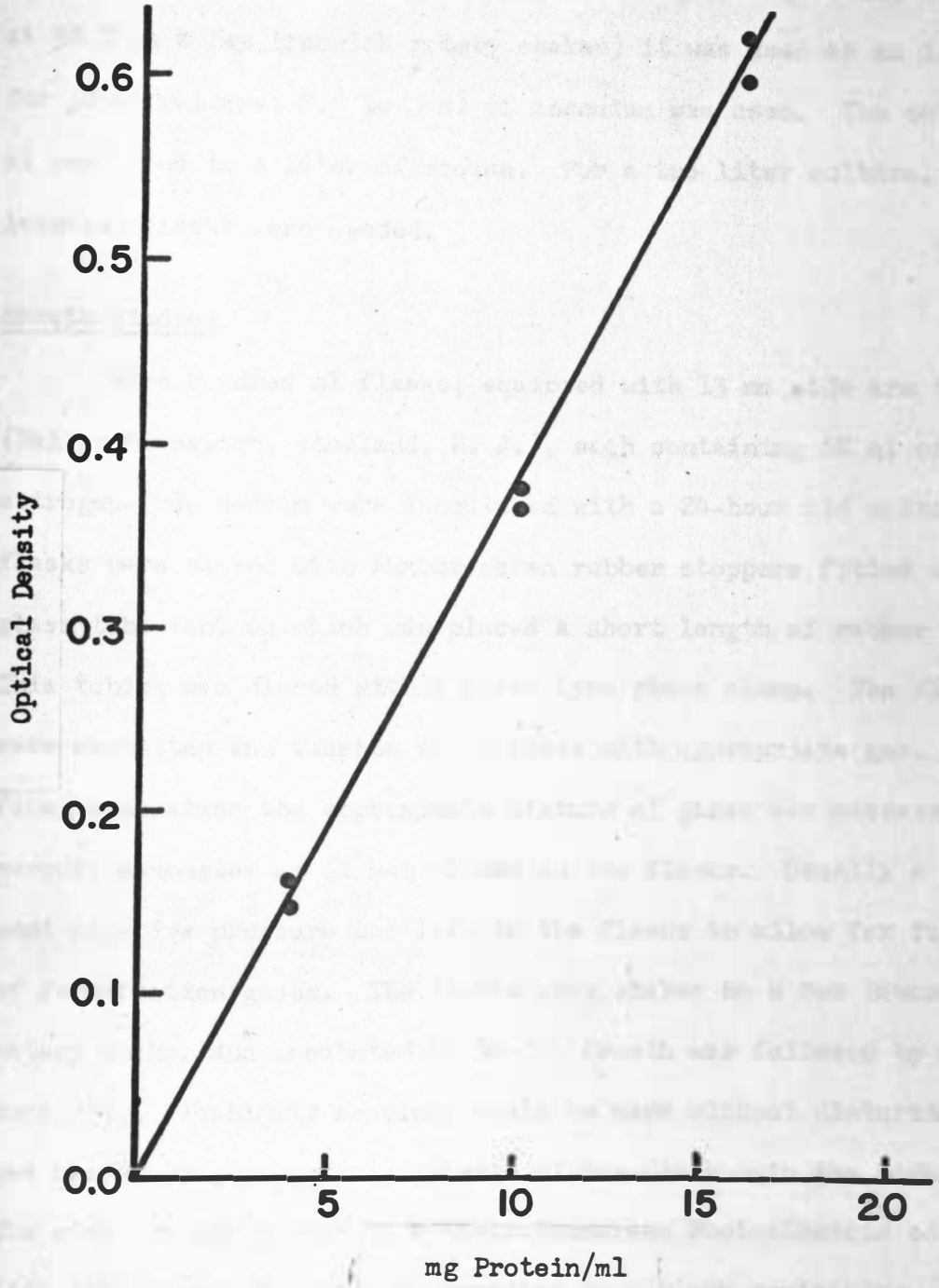

Figure 1. Standard Curve for Biuret Protein Determination. Bovine albumin used as the standard.

culture had reached an exponential rate of growth (usually overnight at 32 C on a New Brunwick rotary shaker) it was used as an inoculum. For 50 ml cultures 2.5 to 5 ml of inoculum was used. The entire 50 ml was added to a liter of medium. For a two liter culture, two inoculum flasks were needed.

Growth studies

Five hundred ml flasks, equipped with 13 mm side arm tubes (Bellco Glassware, Vineland, N. J.), each containing 50 ml of a nitrogen-free medium were inoculated with a 24-hour old culture. The flasks were closed with number seven rubber stoppers fitted with a glass tube vent on which was placed a short length of rubber tubing. This tubing was closed with a screw type pinch clamp. The flasks were evacuated and flushed three times with appropriate gas. On the final evacuation the approximate mixture of gases was measured with a mercury manometer as it was placed in the flasks. Usually a 10 per cent negative pressure was left in the flasks to allow for formation of fermentation gases. The flasks were shaken on a New Brunswick rotary shaker and incubated at 32 C. Growth was followed by measuring turbidity. Turbidity readings could be made without disturbing the gas phase, by pouring the contents of the flask into the side arm. The side arm was placed in a Klett-Summerson Photoelectric colorimeter (650 millimicron filter) and compared to a blank containing uninoculated medium.

Mass cultures

When large numbers of cells were required, a two-liter Erlenmyer flask containing two liters of medium was used. The flask was closed with a three-hole stopper carrying a gas inlet tube attached to a sintered glass dispersion tube, a gas outlet tube and a sampling tube. High purity tank nitrogen gas was slowly bubbled through the culture. Growth could again be followed by turbidity and the cells could be harvested at any point on the growth curve by centrifugation.

Inhibition studies

For inhibition studies a one-liter culture was grown under flowing nitrogen until it was in the exponential phase of growth. Aliquotes could then be removed and placed into 500 ml side arm flasks. The desired experimental atmospheres could then be placed over the culture. When a small quantity of gas, for example 5 ml of acetylene, was to be added to the culture, this was done by means of a syringe and needle. The needle was inserted into the rubber tubing on the glass vent of the stopper. After the gas was added the needle was removed. In this manner the atmosphere over the culture would not have to be disturbed to change the mixture of gas. Turbidity could again be followed as a measure of growth.

Cell-Free Studies

A two-liter mass culture was prepared as described (see procedures for growth studies). The cells were harvested when a reading was reached in the range of 130 - 190 turbidity units on the Klett-Summerson colorimeter. The point of harvest most often used, 160 Klett units, was about the middle of the exponential phase of growth. The Szent-Gyorgyi and Blum continuous flow attachment for the SS-34 Sorvall centrifuge was used for harvesting the cells.

The cells were resuspended in 0.025 M phosphate buffer, pH 7.0. Cells from the two-liter culture were resuspended in 10 ml of the buffer. Nitrogen gas was constantly flushed into the tubes while the cells were being resuspended. This prevented oxygen inactivation of the nitrogenase. The cell suspension was poured into a chilled French pressure cell and immediately broken. Pressure was supplied at 20,000 psi from a Carver hydraulic press. Again, nitrogen gas was flushed into the tube containing the crude extract. The remaining whole cells were removed from the crude extract by centrifugation under an atmosphere of nitrogen in a capped centrifuge tube at 24,000 x g for 30 minutes using a Beckman Model L Ultra-centrifuge equipped with a Type 50 rotor (temperature at 2 C). The supernatant liquid was quickly transferred into a serum bottle, and an atmosphere of nitrogen was placed over the cell-free extract. The extracts, which were dark brown in color, were stored at 4 C.

Mahl (1966) and Mahl and Wilson (1966) reported that ATP, creatine phosphate and creatine phosphokinase were essential for the activity of the cell-free nitrogen fixing system of the Klebsiella pneumoniae extracts. The components they reported were the same as those found by Bulen et al. (1965) for Azotobacter extracts.

Procedure for cell-free nitrogen fixation by Klebsiella pneumoniae:

1. To a 25 ml serum bottle (E. H. Sargent & Co. Number S-9065) add:
   a. 0.05 ml of 0.05 M cacodylate buffer containing 5 micromoles $MgCl_2$.
   b. 0.45 ml of 0.05 M cacodylate buffer containing 2.7 mg ATP, 10.5 mg creatine phosphate and 0.2 mg creatine phosphokinase.
2. Stopper the serum bottle. Evacuate and flush three times and fill with the desired atmosphere. A helium atmosphere is used as the control.
3. Add with a syringe 0.2 ml dithionite ($Na_2S_2O_4$) solution containing 3.5 mg dithionite. This must be prepared fresh daily in 0.2 M cacodylate buffer which is oxygen-free and nitrogen-saturated.
4. Add 0.3 ml of cell-free extract. This brings the final volume of the reaction mixture to 1 ml.
5. The mixture is incubated at 32 C for 30 minutes.

After incubation the serum stoppers are removed and one ml of saturated K_2CO_3 is added. Rubber stoppers holding etched glass rods previously dipped in 5N H_2SO_4 are quickly inserted. Distillation of the ammonia released by the alkali is allowed to proceed overnight at room temperature.

The etched glass rod is then placed in a 50 ml beaker containing 5 ml of distilled water and stirred. After removing the etched glass rod, 3 ml of Nessler's reagent (Johnson, 1941) and 2 ml of 3N NaOH is added. The mixture is stirred and placed into one of a set of matched colorimeter tubes. The mixture is allowed to stand for 15 minutes before the absorbance is measured at 490 millimicrons in a Bausch and Lomb Spectronic 20 spectrophotometer. The ammonia content is read from a standard curve (Figure 2). Micrograms of nitrogen fixed is determined by subtracting the amount of ammonia in the helium controls from those of the nitrogen reaction mixtures.

Procedure for Study of Acetylene Incorporation

The culture of Klebsiella pneumoniae was grown under nitrogen-fixing conditions as described in the mass culture section. Growth was followed by turbidity readings in a Klett-Summerson Photoelectric colorimeter. When turbidity readings reached 130 - 190 Klett units, the cultures were transferred to a 2 l heavy walled suction flask. The flask was equipped with a rubber stopper fitted with a glass tube sealed with a section of rubber tubing and a screw clamp. The side hose connection of the suction flask was sealed with a short piece of rubber tubing and a screw-type clamp.

The appropriate gas atmosphere could be measured into the culture by a mercury manometer. In most experiments 70 per cent nitrogen, 10 per cent acetylene, and 20 per cent vacuum was used.

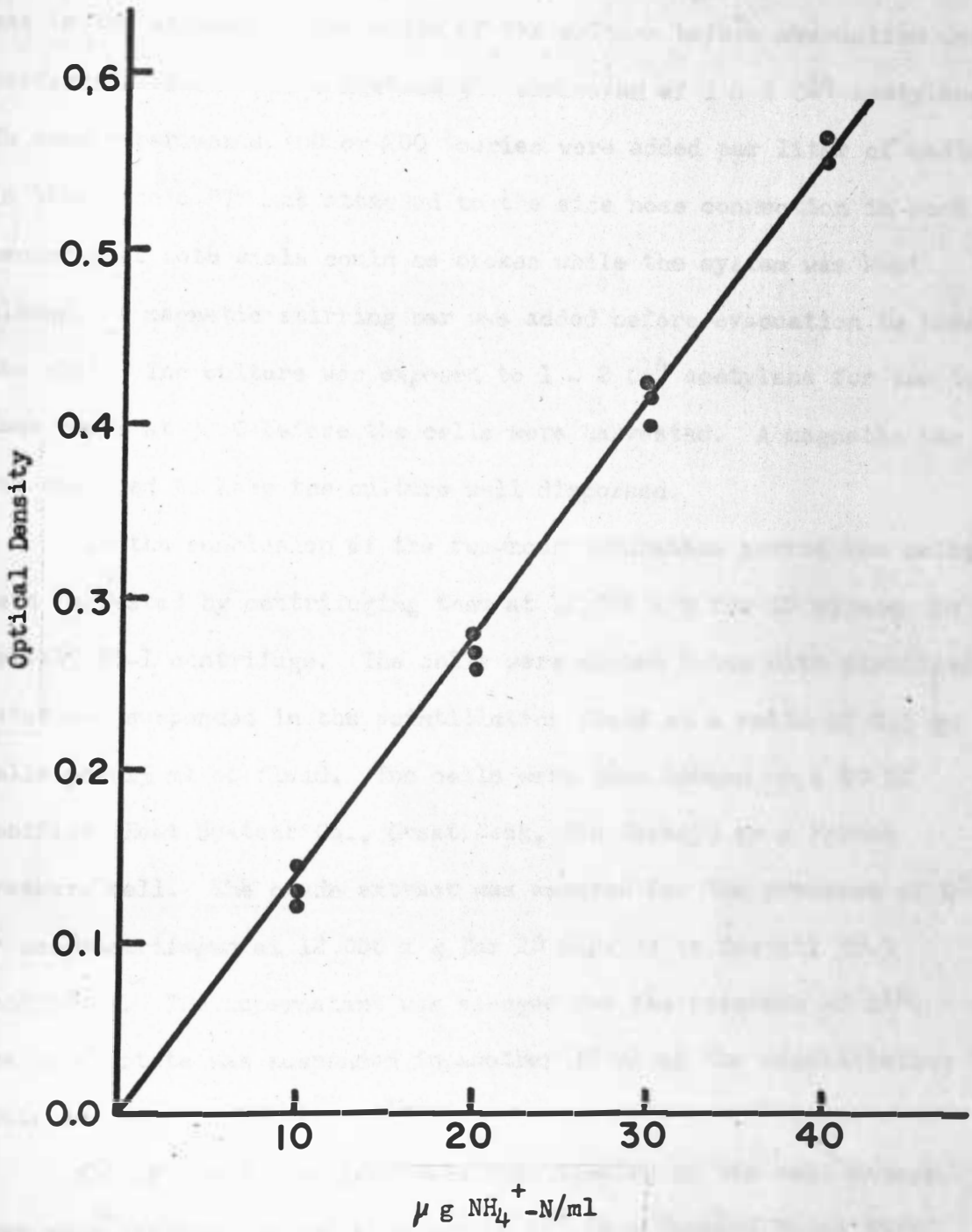

Figure 2. Standard Curve for Ammonium-Nitrogen.

The 1 - 2 C^{14} acetylene was added by attaching the vial of labelled gas to the side-hose connection of the culture before evacuation was performed. Each vial contained 50 curies/mM of 1 - 2 C^{14} acetylene. In some experiments 100 or 200 curies were added per liter of medium. In this case a "T" was attached to the side hose connection in such a manner that both vials could be broken while the system was kept closed. A magnetic stirring bar was added before evacuation to break the vial. The culture was exposed to 1 - 2 C^{14} acetylene for two to four hours at 32 C before the cells were harvested. A magnetic bar was employed to keep the culture well dispersed.

At the conclusion of the two-hour incubation period the cells were harvested by centrifuging them at 12,000 x g for 10 minutes in a Sorvall SS-1 centrifuge. The cells were washed twice with distilled water and suspended in the scintillation fluid at a ratio of 0.5 gm cells per 15 ml of fluid. The cells were then broken by a 20 KC sonifier (Heat Systems Co., Great Neck, New Jersey) or a French pressure cell. The crude extract was assayed for the presence of C^{14} or was centrifuged at 12,000 x g for 20 minutes in Sorvall SS-1 Centrifuge. The supernatant was assayed for the presence of C^{14}. The precipitate was suspended in another 15 ml of the scintillation fluid and then assayed for C^{14}.

All of these fractions were then treated in the same manner. They were checked for the presence of C^{14} in a Packard Model 3324 Tri-Carb Liquid Scintillation Spectrometer (Packard Operation Manual No. 2018).

To determine the quenching of the beta particles, one ml of standard solution (C^{14} dieldrin containing .0036 μcurie/ml) was added to 15 ml of scintillation fluid. The quenching factor was determined.

Each sample was read in the scintillation spectrometer and one ml of dieldrin standard was added to each vial and again read. From this the corrected count of the sample was determined.

In some experiments where turbidity was a problem, Packard Thixotropic Gel Powder (CAB-O-SIL) was added at the rate of 0.6 g per 15 ml scintillation fluid (Rapkin, 1963). The turbidity was evenly dispersed throughout the fluid, therefore making the reading more accurate.

RESULTS AND DISCUSSION

Effect of Acetylene as an Inhibitor of Nitrogen Fixation by Klebsiella pneumoniae Cells

This study was conducted to determine if acetylene would inhibit nitrogen fixation by the facultative anaerobe Klebsiella pneumoniae strain M5al. Schollhorn and Burris (1966) reported acetylene is an inhibitor of nitrogen fixation in Clostridium pasteurianum. Dilworth (1966) reported that acetylene inhibited nitrogen fixation by cell-free extracts of C. pasteurianum. If it could be shown that acetylene acts as inhibitor of nitrogen fixation in other species of nitrogen fixing bacteria, then this could be a major contribution to the understanding of the nitrogen fixation pathway.

In reviewing the work of Pengra and Wilson (1958) it was noted that K. pneumoniae underwent a 12-15 hour lag in growth before fixation of molecular nitrogen (N_2) began. This lag could be explained by the requirement of a nitrogenase induction period. Pengra and Wilson further noted that a "fixed" nitrogen source was not required for nitrogenase induction. Therefore, the cultures were grown either with N_2 as the sole source of nitrogen or with N_2 and 10 μg N/ml as NH_4Cl as a source of nitrogen. When nitrogen fixation began, a 10 per cent atmosphere of acetylene was added to each of these two nitrogen sources and growth was compared to control cultures to which no acetylene had been added (Figure 3). In both of the above types of

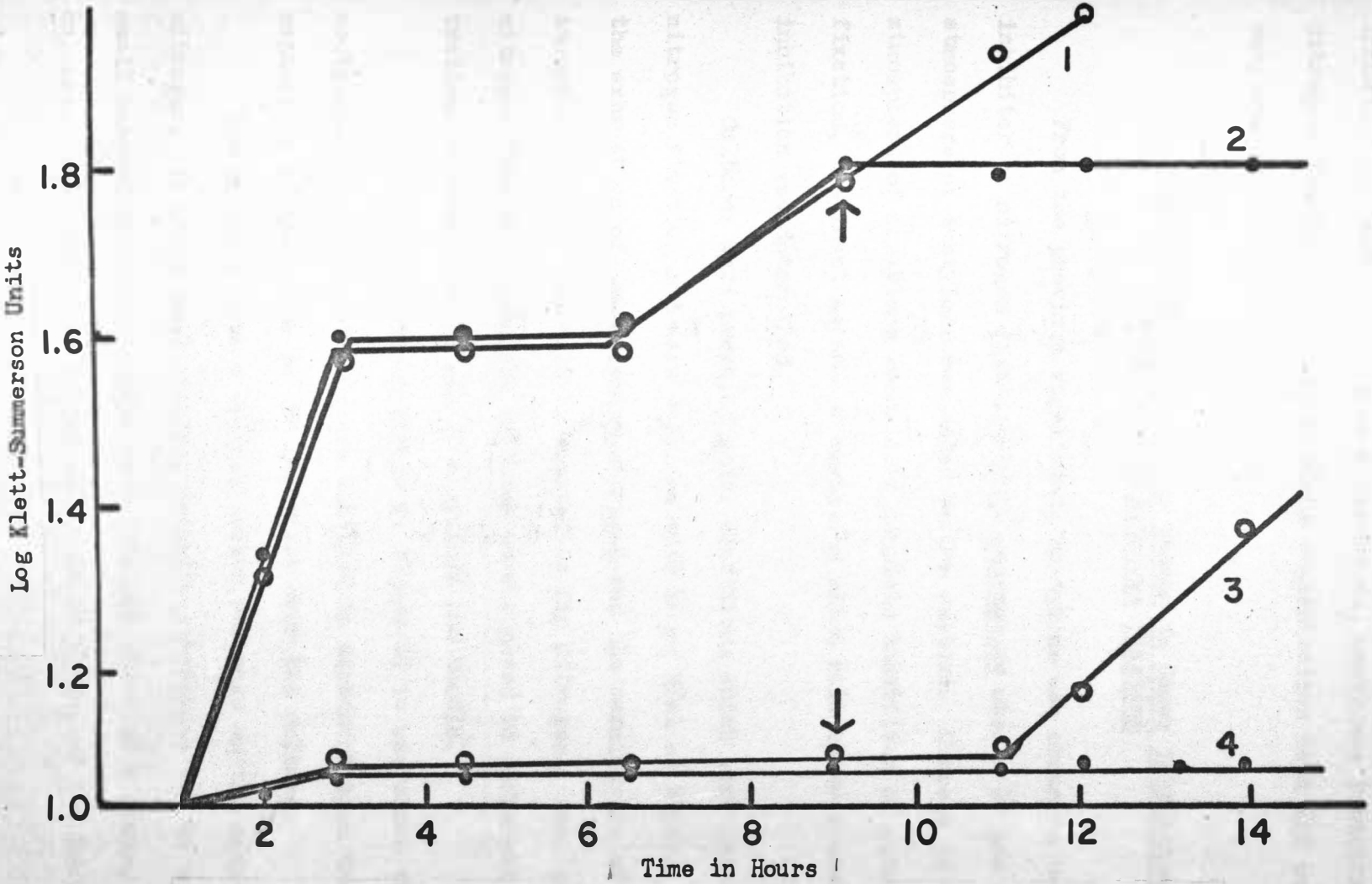

Figure 3. Effect of Acetylene on Nitrogen Fixation by Klebsiella pneumoniae. Curve 1 and 2, nitrogen atmosphere and 10 g N/ml as NH_4Cl. Curve 3 and 4, nitrogen atmosphere. Acetylene, 10%, added to Curve 2 and Curve 4 at 9 hours.

induction periods of nitrogenase formation, acetylene inhibited nitrogen fixation. At the acetylene concentration used (10 per cent), complete inhibition was observed.

Concentration of Acetylene Needed to Cause Inhibition of Whole Cell Nitrogen Fixation

From the previous experiment, acetylene was shown to be an inhibitor of nitrogen fixation by *K. pneumoniae* when a 10 per cent atmosphere of acetylene was added to the culture. Since a 10 per cent atmosphere of acetylene results in complete inhibition of nitrogen fixation, the least amount of acetylene which will cause a measurable inhibition was determined.

Cultures were prepared under conditions which were suitable for nitrogen fixation and were supplied with 10 μg N/ml as NH_4Cl. After the exhaustion of the ammonium-nitrogen and the termination of the induction period, the culture started to fix nitrogen. Soon after the nitrogen fixation began the cultures were exposed to different concentrations of acetylene by use of a syringe and needle.

From this experiment (Table 1; Figure 4) it was shown that the acetylene inhibition of nitrogen fixation is dependent upon the concentration of acetylene in the gas phase over the culture.

The cultures gave a typical growth response on the ammonium-nitrogen. In whole cell studies, definite inhibition can be noted by small concentrations of acetylene in the gas phase of a fixing culture. When the acetylene was added at 12 hours, or two hours after

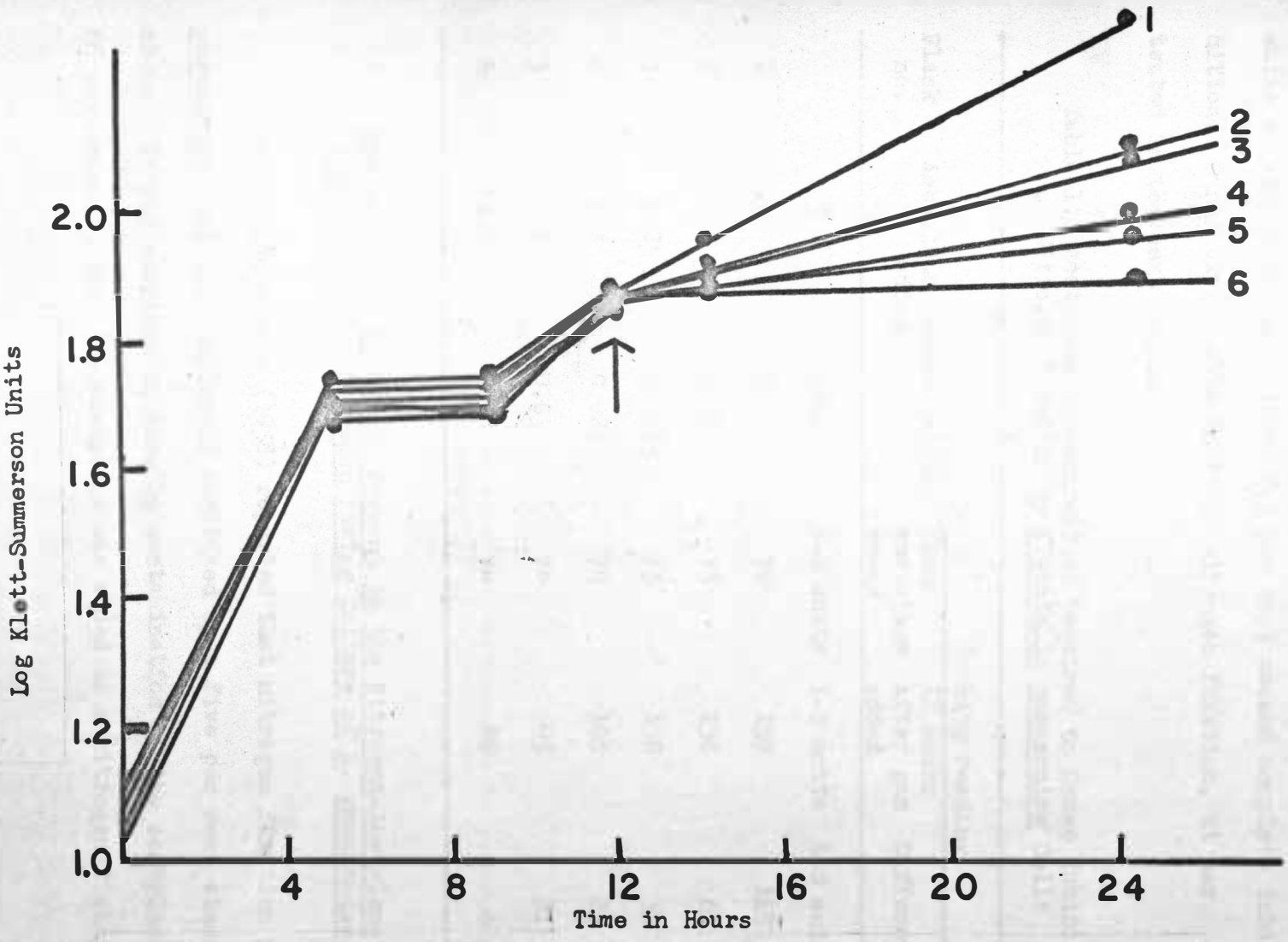

Figure 4. Effect of Varying Concentrations of Acetylene on Nitrogen Fixation by Klebsiella pneumoniae. Acetylene was added at 12 hours: Curve 1, 0.0%; Curve 2, 1.0%; Curve 3, 1.25%; Curve 4, 2.5%; Curve 5, 5.0%; Curve 6, 10.0%.

nitrogen fixation started, an inhibition was noted. An atmosphere containing one per cent acetylene caused about 50 per cent inhibition, while an atmosphere containing 2.5 per cent caused complete inhibition. Since oxygen also inhibits nitrogen fixation, it was tested in the next section.

Table 1. Acetylene Concentrations Required to Cause Inhibition of Nitrogen Fixation by Klebsiella pneumoniae Cells

Flask no.	Acetylene concentration added		Turbity reading		
			When acetylene added	12 hours after gas added	Difference
	%	atm.	K-S units	K-S units	K-S units
1	0	0	79	197	118
2	1.0	0.01	75	136	61
3	1.25	0.0125	75	130	55
4	2.5	0.025	79	108	29
5	5.0	0.05	74	95	21
6	10.0	0.1	74	80	6

Determination if O_2 was Present in the Nitrogen-Acetylene Atmosphere of a Nitrogen Fixing Culture of K. pneumoniae

Pengra and Wilson (1958) reported that nitrogen fixation by K. pneumoniae M5a1 was completely inhibited by a five per cent atmosphere of O_2. It was possible to have O_2 contamination of the acetylene or to introduce O_2 when the acetylene was added to a nitrogen fixing

culture. The tank of acetylene was assayed for O_2 contamination on a gas chromatograph and was found to contain less than 0.1 per cent O_2. Therefore, the complete inhibition of nitrogen fixation by acetylene was not caused by O_2 contamination of the tank acetylene.

An experiment was devised to eliminate the possibility of the introduction of O_2 by the technique of adding the acetylene. One series of flasks received a measured concentration of acetylene in the usual manner. To a duplicate series, a similar concentration of nitrogen was added (Table 2; Figure 5). By comparing the degree of inhibition between the two series of flasks it was shown that the inhibition was not caused by the introduction of O_2 during the addition of acetylene.

Table 2. Effect of the Addition of Nitrogen and Acetylene to a Nitrogen Fixing Culture of K. pneumoniae*

Flask no.	Gas added		Turbity		
			When gas added	8 hours after gas added	Difference
	%		K-S units	K-S units	K-S units
1	1.0	C_2H_2	48	81	33
2	1.0	N_2	47	111	64
3	2.5	C_2H_2	43	54	11
4	2.5	N_2	45	110	65
5	5.0	C_2H_2	43	44	1
6	5.0	N_2	45	105	60

*The culture was grown on 10 g N/ml as NH_4Cl and N_2 gas atmosphere.

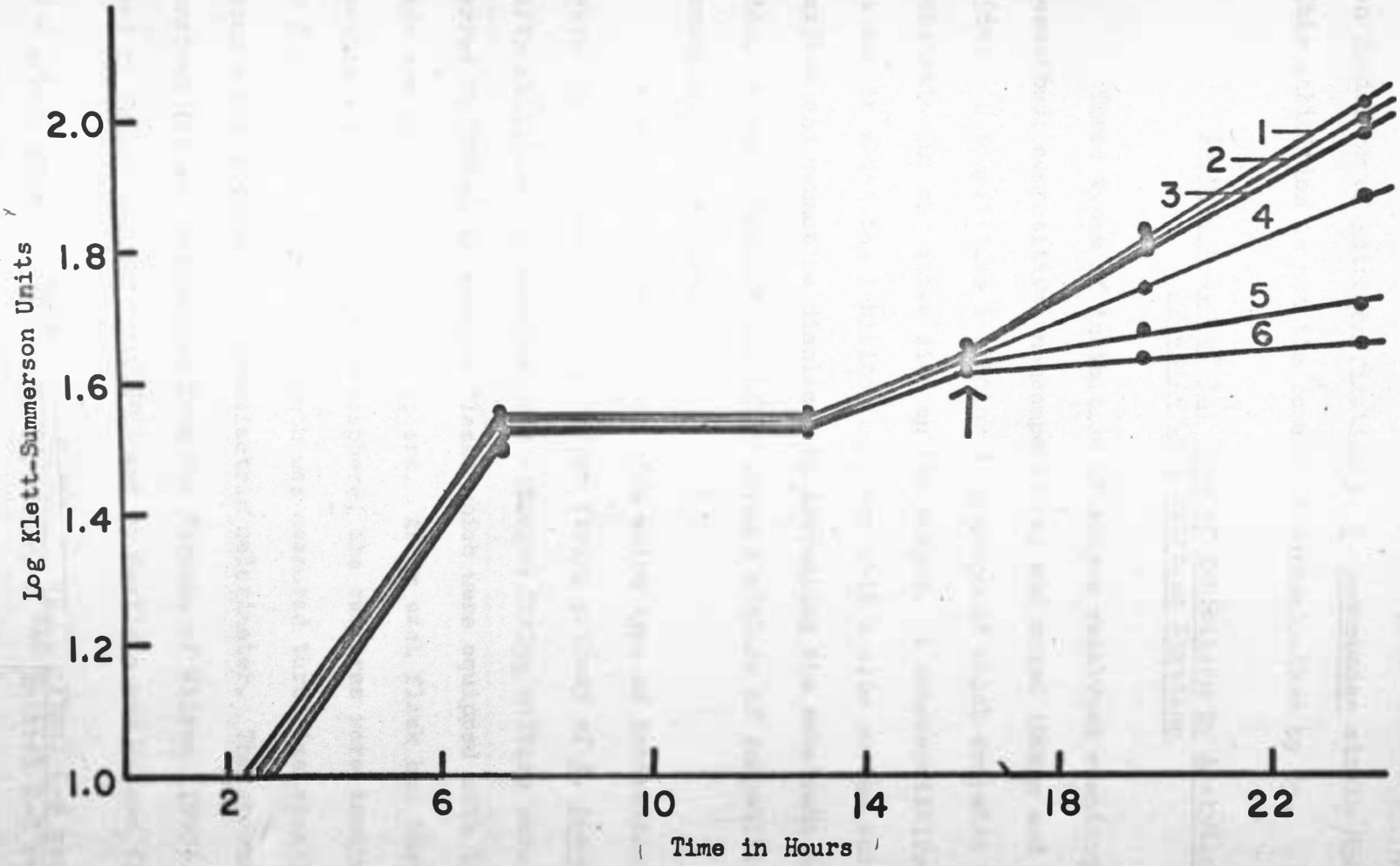

Figure 5. Effect of the Addition of Acetylene and Nitrogen to Nitrogen Fixing Cultures of Klebsiella pneumoniae. Nitrogen was added at 16 hours: Curve 1, 1.0%; Curve 2, 2.5%; Curve 3, 5.0%. Acetylene was added at 16 hours: Curve 4, 1.0%; Curve 5, 2.5%; Curve 6, 5.0%.

From the data thus presented it is apparent that acetylene is an inhibitor of nitrogen fixation in K. pneumoniae strain M5a1 and this inhibition is not the result of contamination by O_2.

Determination of the Type of Inhibition by Acetylene on Whole Cell Nitrogen Fixation

Three types of inhibition of enzyme catalyzed reactions are described; competitive, noncompetitive, and mixed (Dixon and Webb, 1964). A competitive inhibitor is a compound which competes with the substrate for an active site on the enzyme. A noncompetitive inhibitor is one in which the inhibitor combines with a site on the enzyme surface and cannot be displaced by increasing the substrate concentration. A mixed type of inhibitor gives a mixture of competitive and noncompetitive effects.

A study was made to determine which type of inhibition was caused by acetylene in the nitrogen fixing pathway of K. pneumoniae. Fifty milliliter quantities of a nitrogen fixing culture were transferred to 500 ml Erlenmeyer flasks which were equipped with 13 mm side arm tubes and rubber stoppers. After each flask had the desired acetylene and/or nitrogen atmosphere, the cultures were incubated at 32 C on a rotary shaker. Growth was measured turbidimetrically using a Klett-Summerson Photoelectric colorimeter. The growth rate constant (K) was determined from the formula of Wilson (1949) and used by Pengra and Wilson (1958) and by Westlake and Wilson (1959):

$$K = \text{growth rate constant} = \frac{2.303}{\text{Time in hours}} \log_{10} \frac{\text{Final K-S reading}}{\text{Initial K-S reading}}$$

The data and results from this experiment are shown in Table 3 and Figure 6.

Table 3. The Effect of 2.5% Acetylene and Varying Concentrations of Substrate (N_2) on Nitrogen Fixation by Klebsiella pneumoniae*

Flask no.	Acetylene added	Nitrogen added	Reciprocal of the growth rate constant	Reciprocal of the partial pressure of nitrogen
	%	%	1/K	1/p N_2
1	2.5	100	7.52	1.00
2	2.5	100	7.45	1.00
3	2.5	80	15.01	1.25
4	2.5	80	16.69	1.25
5	2.5	60	18.87	1.67
6	2.5	60	15.02	1.67
7	2.5	40	27.25	2.50
8	2.5	40	28.74	2.50
9	2.5	20	34.36	5.00
10	2.5	20	---	---
11	0	100	7.39	1.00
12	0	80	8.22	1.25
13	0	60	7.59	1.67
14	0	40	10.7642	2.50

*The remaining gas phase was adjusted to one atmosphere with helium.

Figure 6 is a Lineweaver-Burk double reciprocal plot as described by Wilson (1949) and by Dixon and Webb (1964). The reciprocals of the growth rate constants (1/K) were plotted against the reciprocals of the partial pressures of nitrogen because this yielded a straight line. From the slopes and the intercepts of the lines, the type of inhibition was determined. The slopes of the lines were

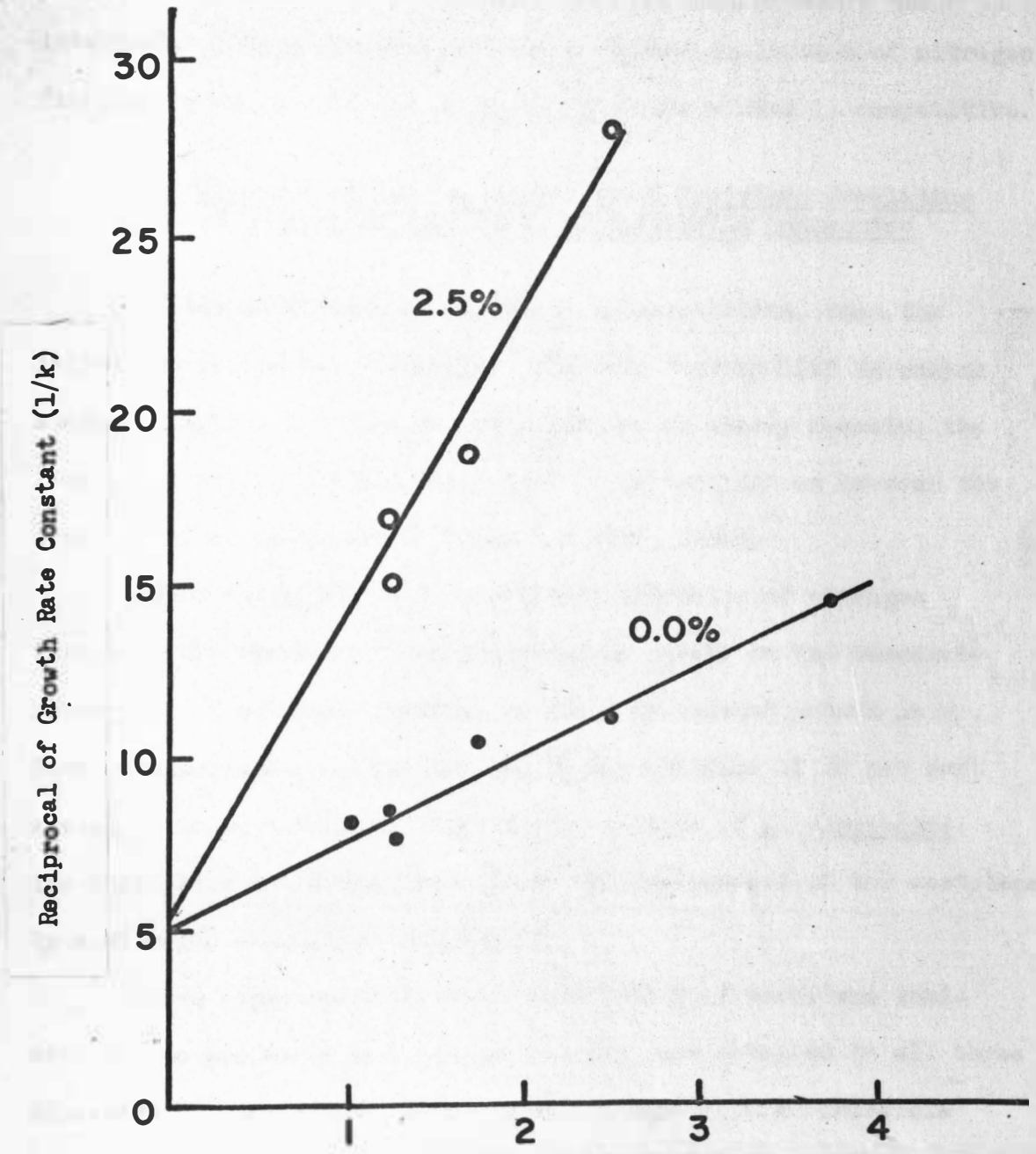

Figure 6. Lineweaver-Burk Double Reciprocal Plot of 2.5% Acetylene Inhibition of Nitrogen Fixation by Klebsiella pneumoniae.

significantly different. The lines had the same ordinate and intercept. This indicates that the acetylene inhibition of nitrogen fixation by whole cells of K. pneumoniae strain M5al is competitive.

Determination of the Reversibility of Acetylene Inhibition of Nitrogen Fixing Cells of Klebsiella pneumoniae

If the inhibition of acetylene is competitive, then the inhibition should be reversible. The term "reversible" in enzyme studies "implies that the activity returns on merely removing the free inhibitor... showing that there is an equilibrium between the free inhibitor and enzyme" (Dixon and Webb, 1964).

Since acetylene is a competitive inhibitor of nitrogen fixation, the removal of acetylene should result in the immediate resumption of nitrogen fixation as shown by renewed growth on N_2. Such an experiment was carried out by the addition of 10 per cent acetylene to an active, nitrogen fixing culture of K. pneumoniae. The inhibition noted was reversed by the replacement of the acetylene by a nitrogen atmosphere (Figure 7).

Three experiments on the reversibility of acetylene inhibition were performed and similar results were obtained in all three experiments. Therefore, acetylene was shown to be a reversible inhibitor of nitrogen fixation by K. pneumoniae strain M5al.

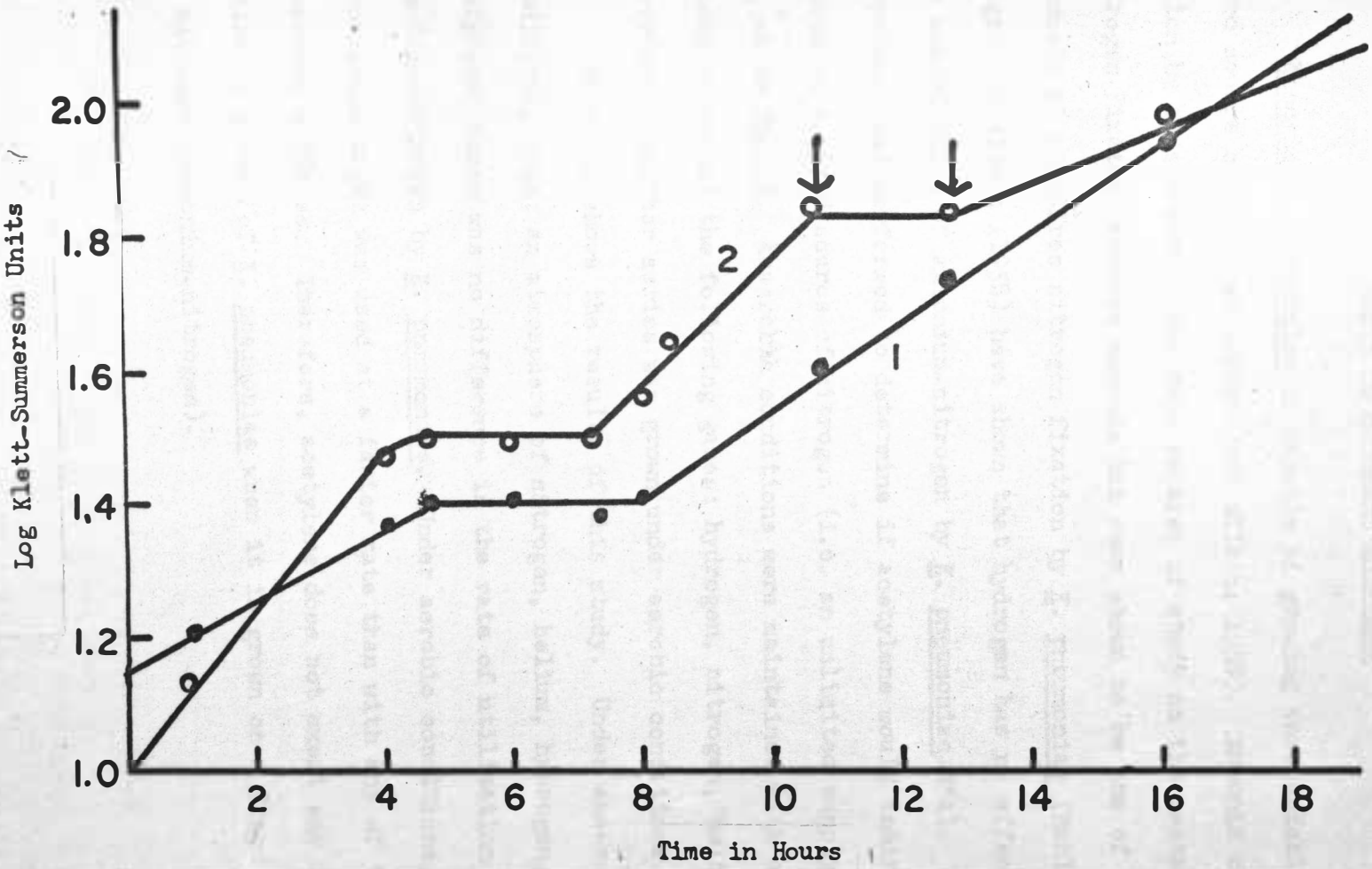

Figure 7. Reversibility of Acetylene Inhibition of Nitrogen Fixation by Klebsiella pneumoniae. Curve 1, nitrogen control; Curve 2, acetylene, 10%, was added at 10 hours and replaced by nitrogen at 12 hours.

Effect of Acetylene on Klebsiella pneumoniae Cells Grown on Ammonium-nitrogen

Klebsiella pneumoniae is capable of growing anaerobically on a fixed source of nitrogen (Pengra and Wilson, 1958). Ammonia utilization by this organism has been an area of study on the mechanism of nitrogen fixation because ammonia has been shown to be one of the end products of cell-free nitrogen fixation by K. pneumoniae (Mahl, 1966). Pengra and Wilson (1958) have shown that hydrogen has no effect on the assimilation of ammonium-nitrogen by K. pneumoniae M5a1. An experiment was performed to determine if acetylene would inhibit growth on a fixed source of nitrogen (i.e. an unlimited supply of NH_4^+-N as NH_4Cl). Anaerobic conditions were maintained with an atmosphere of one of the following gases: hydrogen, nitrogen, helium, or acetylene. Another series was grown under aerobic conditions.

Figure 8 shows the results of this study. Under anaerobic conditions, using an atmosphere of nitrogen, helium, hydrogen, or acetylene, there was no difference in the rate of utilization of ammonium-nitrogen by K. pneumoniae. Under aerobic conditions, ammonium-nitrogen was used at a faster rate than with any of the anaerobic cultures. Therefore, acetylene does not exert any inhibition of growth of K. pneumoniae when it is grown on a fixed source of nitrogen (ammonium-nitrogen).

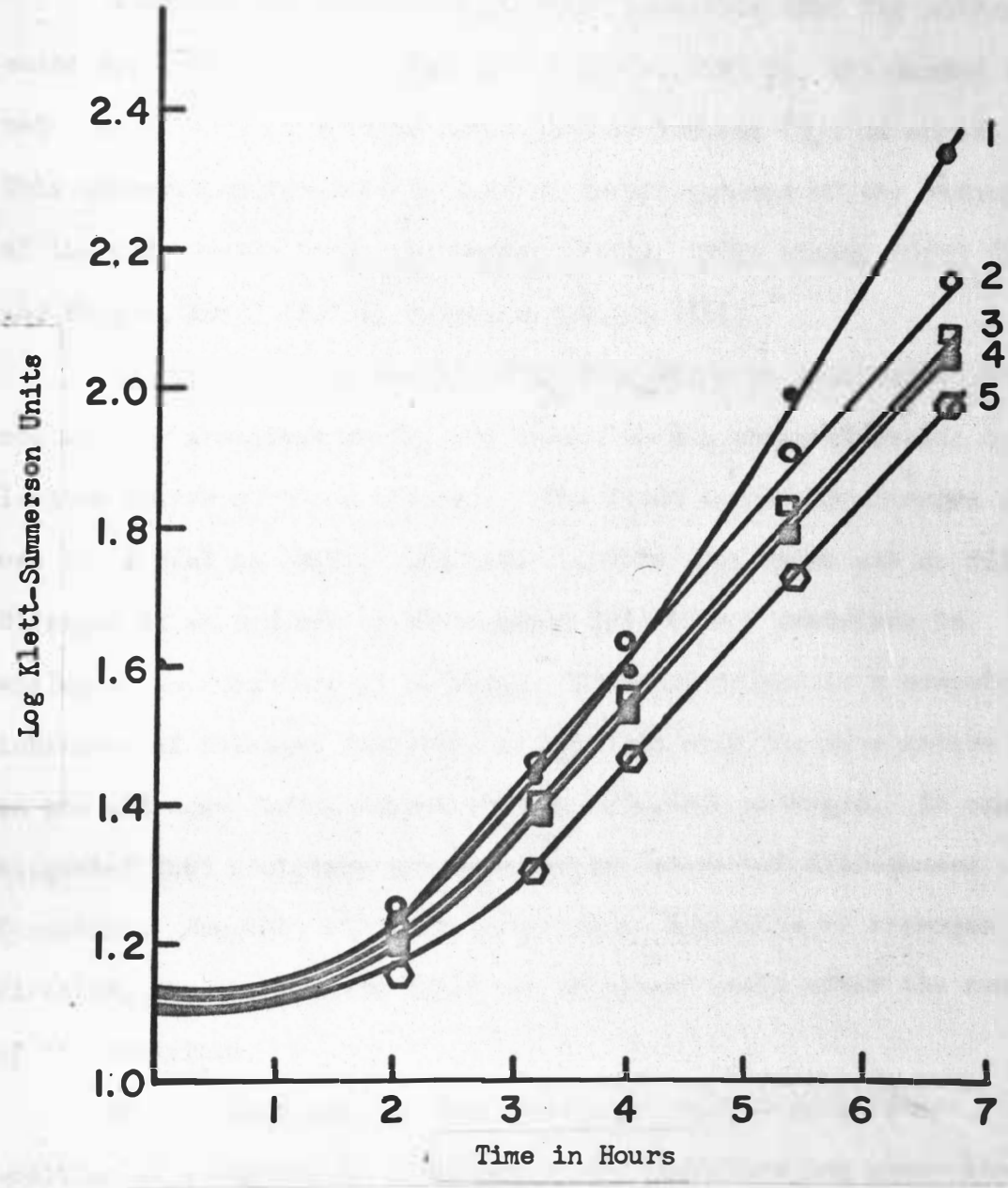

Figure 8. Effect of Nitrogen, Helium, Hydrogen, and Acetylene on the Utilization of Ammonium-Nitrogen as a Sole Nitrogen Source by Klebsiella pneumoniae. Gaseous atmosphere: Curve 1, air; Curve 2, nitrogen; Curve 3, helium; Curve 4, hydrogen; Curve 5, acetylene.

Effect of Acetylene on Nitrogenase Induction in Whole Cells of Klebsiella pneumoniae

Hamilton and Wilson (1955) first posulated that the nitrogenase system of K. pneumoniae is inducible; that is, the enzyme is not present in a detectable form until an inducer (N_2) is added. This observation resulted in several investigations of the induction of the nitrogenase in K. pneumoniae (Patil, 1963) (Yoch, 1965) (Yoch and Pengra, 1966) (Patil, Pengra and Yoch, 1967).

Experiments were conducted in this study in which nitrogen was replaced by acetylene during the inductive lag phase of growth on a limited source of fixed nitrogen. The fixed source of nitrogen used was 10 g N/ml as NH_4Cl. The basis of this experiment was as follows: Nitrogen is an inducer of nitrogenase formation. Acetylene is analogous in structure to nitrogen. Since acetylene is a competitive inhibitor of nitrogen fixation, it combines with the same active site on the nitrogen fixing enzyme as does molecular nitrogen. It can be suggested that acetylene may serve as an inducer of nitrogenase formation. However, since acetylene is an inhibitor of nitrogen fixation, the nitrogenase would not be active until after the removal of the acetylene.

If the acetylene can serve as an inducer of nitrogenase, the addition of acetylene to a culture in the inductive lag phase should not result in an extension of the induction lag when these cultures are compared to a control series to which acetylene was added. Figures 9 and 10 show the results obtained from such an experiment.

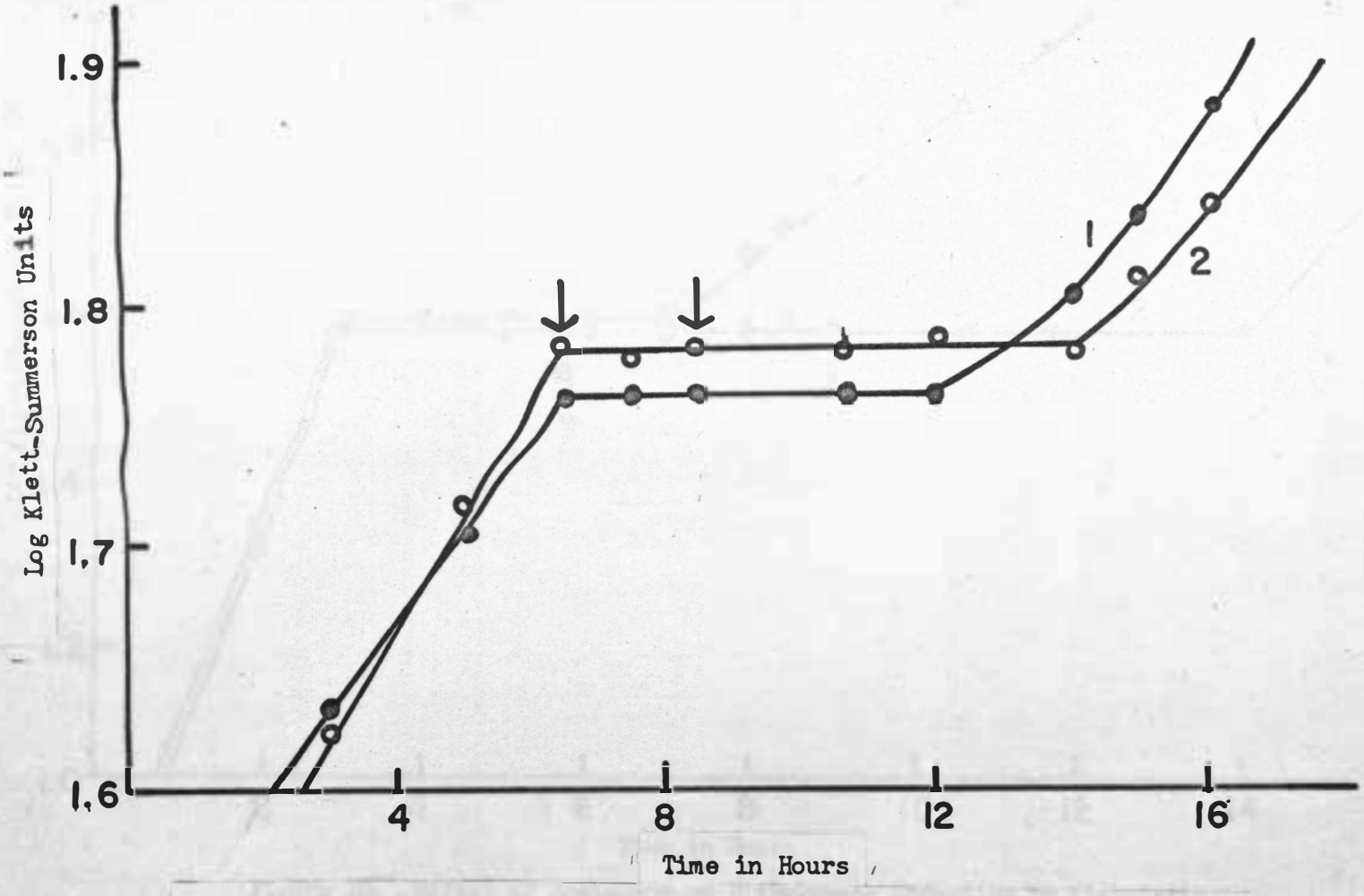

Figure 9. Effect of Acetylene on Nitrogenase Induction by Klebsiella pneumoniae. Curve 1, nitrogen control; Curve 2, acetylene, 100%, added at 6.25 hours and replaced by nitrogen at 8.25 hours.

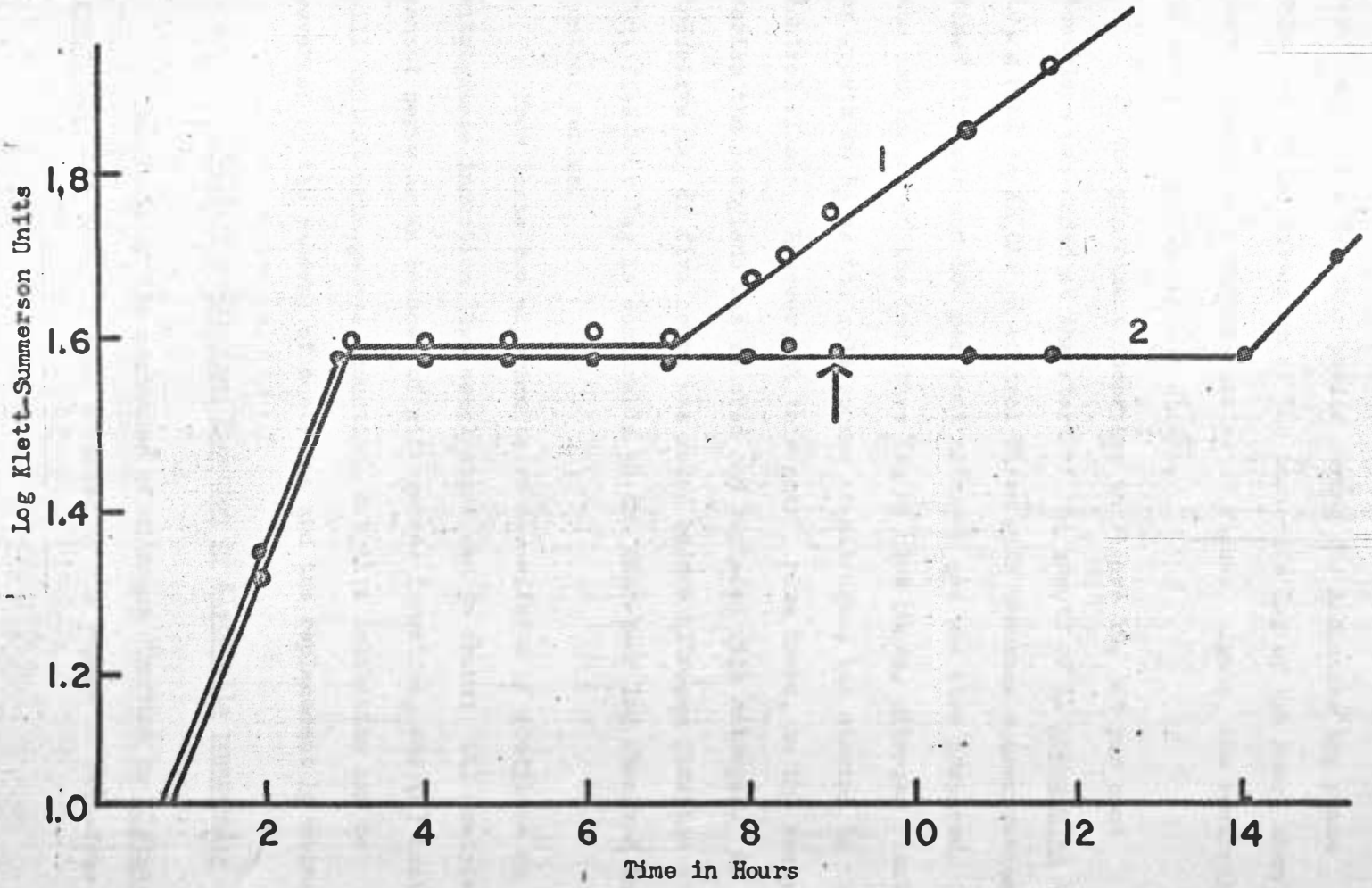

Figure 10. Effect of Acetylene on Nitrogenase Induction by Klebsiella pneumoniae. Curve 1, nitrogen control; Curve 2, acetylene, 100%, until replaced by nitrogen at 9 hours.

It can be noted from Figure 9 that the addition of 100 per cent acetylene for a two-hour period during the inductive lag phase resulted in the extension of the inductive lag by two hours when they were compared to a control series of flasks. Again, the reversibility of acetylene inhibition was observed.

In the experiment described in Figure 10, 100 per cent acetylene was added at the start of the growth of K. pneumoniae on 10 μ g N/ml as NH_4Cl. A control flask with the same concentration of fixed nitrogen and 100 per cent nitrogen gas was also prepared. At the conclusion of the inductive lag of nine hours, nitrogen fixation, or growth on N_2 as the sole source of nitrogen, had started in the control flasks. However, at this point, nine hours, in the acetylene series the acetylene was removed and replaced with nitrogen. An inductive lag of five hours was noted before nitrogen fixation started. This five-hour lag is comparable to the four-hour lag observed in the control series.

From these two experiments on the effects of acetylene on nitrogenase induction, two conclusions can be drawn: (1) acetylene cannot serve as an inducer of nitrogenase formation, and (2) acetylene will inhibit nitrogenase induction, but this inhibition can be reversed by the removal of acetylene and the replacement by nitrogen.

Cell-Free Nitrogen Fixation by Klebsiella pneumoniae

The study of the mechanism of nitrogen fixation by Klebsiella pneumoniae has recently been advanced by the report of cell-free

nitrogen fixation by Mahl (1966) and Mahl and Wilson (1966). Using the methods they described, experiments were conducted to confirm their results and to establish a basis for further studies with acetylene inhibition. Experimental details are shown in the section Cell-Free Studies, Materials, and Methods. The results obtained were 38 μg NH_4^+-N fixed per hour by an extract which contained 12 mg protein per ml. The calculated specific activity was about 3. This compares with a specific activity reported by Mahl (1966) which ranged from 3 to 5. This experimental result verifies the above report of cell-free nitrogen fixation by K. pneumoniae strain M5a1.

Effect of Acetylene on Cell-Free Nitrogen Fixation by Klebsiella pneumoniae

A study was next undertaken to determine if acetylene could act as an inhibitor of cell-free nitrogen fixation by K. pneumoniae as was previously demonstrated with whole cells. Since an atmosphere of 2.5 per cent resulted in complete inhibition of whole cell nitrogen fixation, 2.5 per cent acetylene was selected as a concentration for cell-free inhibition studies. The results given in Table 4 show that 2.5 per cent acetylene is an inhibitor of cell-free nitrogen fixation by K. pneumoniae.

Of the two sets of controls, the ones with nitrogen substrate showed 10 and 12 μg NH_4^+-N fixed per ml. In the series to which 2.5 per cent acetylene was added, no fixation of nitrogen could be detected. Therefore, a concentration of 2.5 per cent acetylene

causes complete inhibition of cell-free nitrogen fixation. Further experiments were needed to determine the least amount of acetylene which could cause inhibition.

Table 4. Effect of Acetylene on Cell-Free Nitrogen Fixation by Klebsiella pneumoniae*

Acetylene	Substrate	Total NH_4^+-N	Fixed NH_4^+-N
%		μg/ml	μg/ml
0.0	N_2	33	10
0.0	N_2	35	12
2.5	N_2	10	0
2.5	N_2	20	0
0.0	He	23	0
0.0	He	22.5	0

*Protein 9.2 mg/ml.

Effect of Varying Concentrations of Acetylene on Cell-Free Nitrogen Fixation by Klebsiella pneumoniae

The previous experiment demonstrated that acetylene is an inhibitor of cell-free nitrogen fixation and 2.5 per cent acetylene resulted in complete inhibition. Experimental results are shown in Table 5 and Figure 11 in which different amounts of acetylene (0 to 2%) were added. The amount of inhibition was determined by comparing the amount of fixation noted with the N_2 and He controls.

Table 5. Effect of Varying Concentration of Acetylene on Cell-Free Nitrogen Fixation by Klebsiella pneumoniae M5al*

Acetylene	Substrate	Total NH_4^+-N	Fixed NH_4^+-N
%		µg/ml	µg/ml
2.0	N_2	27	4
2.0	N_2	33	10
1.0	N_2	28	5
1.0	N_2	27	4
0.5	N_2	37	14
0.5	N_2	37	14
0.0	N_2	47	24
0.0	N_2	48	25
0.0	He	24	0
0.0	He	23	0

*Protein 8.5 mg/ml.

Inhibition by acetylene of cell-free fixing extracts is shown to be a direct function of the partial pressure of acetylene (Figure 11). One half of one per cent acetylene in the atmosphere resulted in 55 per cent inhibition and complete inhibition is noted with one per cent acetylene. These quantities are similar to those required for inhibition of cell-free extracts of Clostridium pasteurianum (Dilworth, 1966).

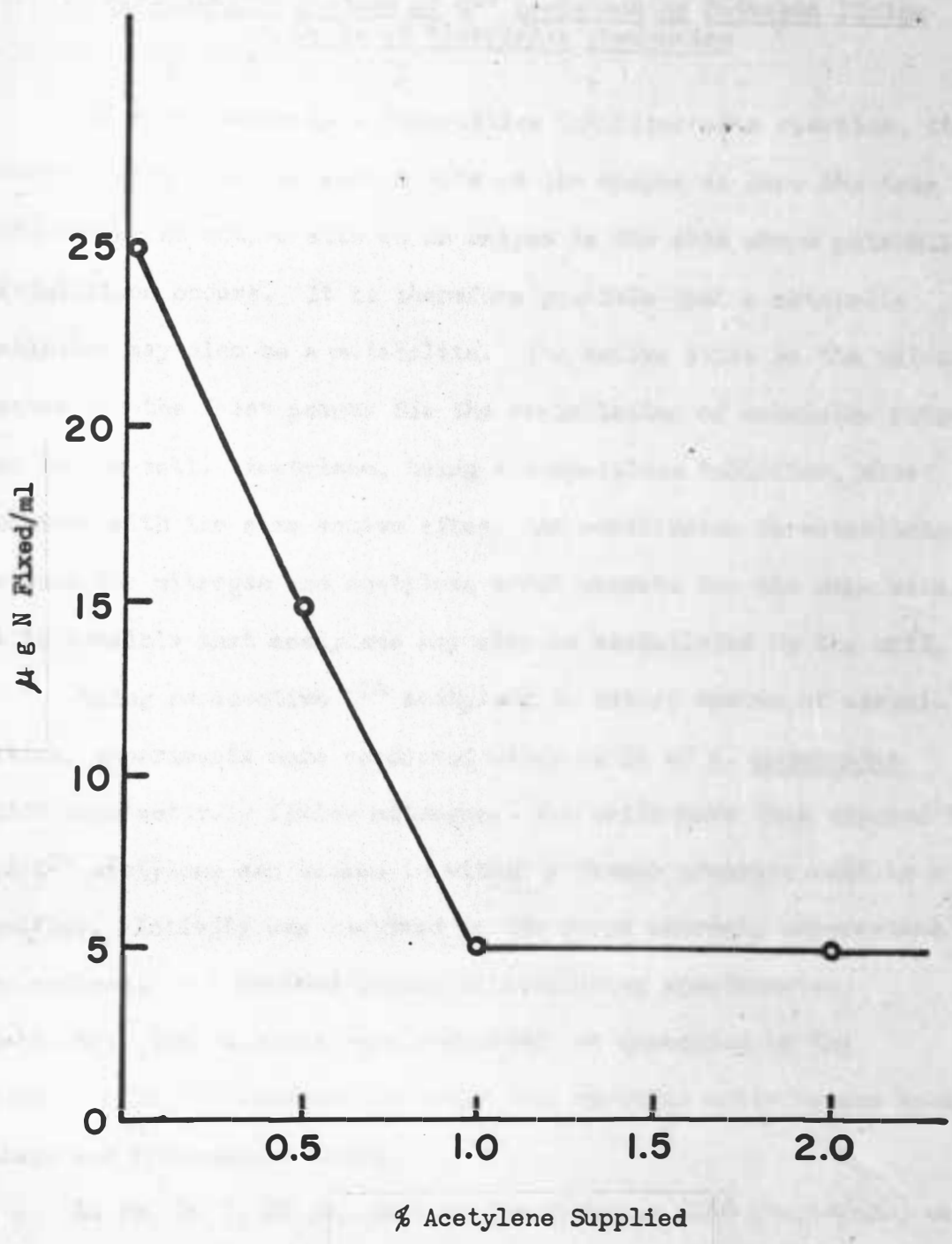

Figure 11. Effect of Concentration of Acetylene on Nitrogen Fixation by Cell-Free Extracts of Klebsiella pneumoniae.

Assimilation Studies of C^{14} Acetylene by Nitrogen Fixing Cells of Klebsiella pneumoniae

If a substance is a competitive inhibitor of a reaction, it combines with the same active site on the enzyme as does the true substrate. An active site on an enzyme is the site where metabolic assimilation occurs. It is therefore possible that a metabolic inhibitor may also be a metabolite. The active sites on the nitrogenase are the first points for the assimilation of molecular nitrogen by the cell. Acetylene, being a competitive inhibitor, also combines with the same active sites. An equilibrium is established between the nitrogen and acetylene which compete for the same site. It is possible that acetylene may also be assimilated by the cell.

Using radioactive C^{14} acetylene to detect traces of assimilation, experiments were conducted using cells of K. pneumoniae which were actively fixing nitrogen. The cells were then exposed to 1,2 C^{14} acetylene and broken in either a French pressure cell or a sonifier. Activity was compared in the crude extract, supernatant and sediment in a Packard Liquid scintillation spectrometer (Table 6). The readings were corrected for quenching by the addition of a C^{14} standard for which the specific activity was known (Chase and Rabinowitz, 1962).

In sample 1, 68 per cent of the activity (266 counts/min) was located in the supernatant. One-half of this activity was found in sample 2, a heated control. It is not possible to compare sample 1 directly with sample 2 because sample 1 was not corrected for

Table 6. Studies on the Assimilation of C^{14} Acetylene by Klebsiella pneumoniae*

Sample no.	Method of cell breakage	Heat Treatment	C^{14} acetylene added per gram of cells wet weight	Counts/min Centrifuged (12,000 x g for 10 min) Supernated Counts per min	Supernated % activity	Sediment Counts per min	Sediment % activity
1	Sonifier	None	200	266**	68	124**	32
2	Sonifier	Autoclaved	100	60	--	--	--
3	Sonifier	None	100	39	--	--	--
4	Sonifier	None	200	217	81	51	19
5	Sonifier	Autoclaved	200	472	--	--	--
6	French Press	None	200	39***	58	28***	42
7	French Press	Autoclaved	200	50***	67	25***	33

*All readings were corrected for background count.
**Readings were not corrected for a quenching factor.
***Samples were mixed with Thixotropic Gel Power (0.6 g/16 ml.)

quenching. The activity of sample 2, heated, was compared to 3, unheated. Sample 2 has almost twice the activity of 3. Similar results were noted when sample 4, not heated, was compared to sample 5, heated. The author has no explanation for the higher activity in the autoclaved samples. In sample 4 the activity was 81 per cent in the supernatant compared to 19 per cent in the sediment.

In all of the above samples, nonuniform distribution was a factor. To equally disperse the sample, samples 6 and 7 were mixed with Thixotropic Gel Power. Sample 6 was not heated and had 39 counts/min in the supernatant as compared to a heated control, 7, which had 50 counts/min in the supernatant. In the sediments, sample 6 had 28 counts/min while sample 7 had 25 counts/min.

The differences in the radioactive counts between heated and nonheated samples were not great enough to indicate assimilation of C^{14} acetylene by nitrogen fixing K. pneumoniae cells. This experiment was not carried beyond the preliminary stage because adequate safeguards were not available to insure safety during the cell fractionation procedures. Further studies using 1,2 C^{14} acetylene should be made before definite conclusions are made on the assimilation of acetylene.

CONCLUSIONS

(1) Acetylene is an inhibitor of nitrogen fixation by Klebsiella pneumoniae strain M5a1.

(2) The inhibition by acetylene was shown to be a direct function of the partial pressure of acetylene supplied.

(3) In whole cell studies, an atmosphere containing one per cent acetylene caused about 50 per cent inhibition; an atmosphere containing 2.5 per cent caused complete inhibition.

(4) One half of one per cent acetylene in the atmosphere of a cell-free nitrogen fixing extract caused 50 per cent inhibition; an atmosphere containing one per cent caused complete inhibition.

(5) Acetylene inhibition of nitrogen fixation by K. pneumoniae is competitive.

(6) Acetylene inhibition of nitrogen fixation is reversible.

(7) Acetylene did not inhibit growth of K. pneumoniae on ammonium-nitrogen.

(8) Acetylene will not serve as an inducer of nitrogenase formation under the condition tested.

(9) Acetylene will inhibit nitrogenase induction.

(10) No assimilation of 1,2-C^{14} acetylene by nitrogen fixing cells of K. pneumoniae could be detected.

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